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Inhibition of Caspase-3 Improves Contractile Recovery of Stunned Myocardium, Independent of Apoptosis-Inhibitory Effects

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OBJECTIVES The aim of this study was to investigate whether the caspase-3 inhibitor Ac-DEVD-CHO functionally improves stunned myocardium.

BACKGROUND Degradation of troponin I contributes to the pathogenesis of myocardial stunning, whereas the role of apoptosis is unknown. Caspase-3 is an essential apoptotic protease that is specifically inhibited by Ac-DEVD-CHO.

METHODS Isolated working hearts of rats were exposed to 30 min of low-flow ischemia, followed by 30 min of reperfusion. Ac-DEVD-CHO (0.1 to 1 μmol/l) was added 15 min before ischemia/reperfusion or 5 min before reperfusion. Cardiac output, external heart power, left ventricular (LV) developing pressure and contractility (dp/dt_{max}) were measured. Apoptosis was assessed by TUNEL staining and internucleosomal deoxyribonucleic acid fragmentation. Caspase-3 processing and troponin I cleavage were determined by immunoblotting. Caspase-3 activity was measured using a fluorogenic substrate.

RESULTS The addition of Ac-DEVD-CHO before ischemia/reperfusion or before reperfusion dose-dependently and significantly ($p < 0.05$) improved post-ischemic recovery of cardiac output, external heart power, LV developing pressure and dp/dt_{max}, compared with the vehicle (0.01% dimethyl sulfoxide). Ac-DEVD-CHO was similarly effective when given before reperfusion. Ac-DEVD-CHO blocked ischemia/reperfusion-induced caspase-3 activation, but cardiomyocyte apoptosis was unaffected. Troponin I cleavage was not inhibited by Ac-DEVD-CHO.

CONCLUSIONS Caspase-3 is activated in stunned myocardium. Inhibition of caspase-3 by Ac-DEVD-CHO significantly improves post-ischemic contractile recovery of stunned myocardium, even when given after the onset of ischemia. The mechanism(s) of protection by Ac-DEVD-CHO appear to be independent of apoptosis. Inhibition of caspase-3 is a novel therapeutic strategy to improve functional recovery of stunned myocardium. (J Am Coll Cardiol 2001;38: 2063–70) © 2001 by the American College of Cardiology

A significant reduction of coronary blood flow causes myocardial ischemia and contractile dysfunction. The cardiac dysfunction often persists for days, even after coronary artery perfusion has been re-established. This scenario of reversible post-ischemic contractile dysfunction is known as myocardial stunning (1). Depressed contractility, with reduced maximal force generation, is a hallmark of stunning (2–5).

Apoptosis is an evolutionary conserved process of programmed cell death in response to diverse stimuli, such as cardiac development or hypoxia (6). Increased cardiomyocyte apoptosis has been reported in patients with heart failure and experimental myocardial infarction (MI) (7–10). The caspase family of cellular proteases initiates and executes apoptotic cell death (11). Caspase-3, a pivotal effector caspase, is an essential protease of the apoptotic machinery. Caspase-3 proteolytically cleaves a number of death substrates and activates endonucleases, leading to internucleosomal deoxyribonucleic acid (DNA) fragmentation, a hallmark of apoptosis (12). Individual caspases differ in their substrate recognition sequences, which has allowed generation of inhibitors like Ac-DEVD-CHO that specifically inhibit caspase-3 (3). Ac-DEVD-CHO is a tetrapeptide (Asp-Glu-Val-Asp) based on the caspase-3 substrate recognition motif, with an acetylate group coupled to its N-terminal (for enhanced chemical stability) and an aldehyde group conjugated to its C-terminal (for irreversible inactivation of the caspase-3 catalytic cysteine residue) (13,14).

In a rabbit and rat model of MI, administration of a broad-spectrum caspase inhibitor before infarction significantly reduced the infarct size and rate of apoptotic cells in the area at risk (9,10). However, it is unknown whether caspase inhibitors functionally improve stunned myocardium and whether they are effective when given after the onset of myocardial ischemia. The latter would be more relevant to the clinical situation.

Therefore, we investigated the effects of the caspase-3 inhibitor Ac-DEVD-CHO in an isolated working-heart rat model of myocardial stunning. Our data suggest that inhibition of caspase-3 reduces myocardial stunning when

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Abbreviations and Acronyms
DMSO = dimethyl sulfoxide
DNA = deoxyribonucleic acid
LV = left ventricular
LVP = left ventricular pressure
MI = myocardial infarction

initiated after the onset of ischemia (I), but before reperfusion (R). Thus, caspase-3 inhibition represents a novel therapeutic strategy to improve contractile functional recovery of stunned myocardium.

METHODS

Isolated working-heart rat model. Male, 4- to 6-week-old Sprague Dawly rats were anesthetized with pentobarbital (60 mg/kg intraperitoneally [IP]), heparinized (500 IU/100 g body weight IP). The hearts were removed, and the aorta was mounted onto a 1.4-mm cannula and attached to a perfusion apparatus (Hugo Sachs Electronic). The hearts were perfused with oxygenated (95% oxygen, 5% carbon dioxide), noncirculating Tyrode's solution (in mmol/l): 124.6 NaCl; 4.0 KCl; 2.2 $CaCl_2$; 1.1 $MgCl_2$; 24.9 $NaHCO_3$; 0.3 NaH_2PO_4; and 11.1 glucose (pH 7.4) at a perfusion pressure of 51 mm Hg. After equilibration for 15 min, the perfusion was switched to the anterograde working-heart mode, with a preload of 11 mm Hg and an afterload of 51 mm Hg. Aortic pressure was measured through a pressure transducer (Hugo Sachs Electronic). Left ventricular pressure (LVP) was measured by using a microtipped catheter (SPR 407, 2F, Millar Instruments). The maximal rise in LVP was obtained with an electronic differentiation system (PLUGSYS, Hugo Sachs Electronic). Aortic and coronary flow rates were measured by transonic flow probes (Transonic Systems). Heart rate was monitored by electrography (Hugo Sachs Electronic), and the hearts were paced at 5 Hz.

Experimental groups and protocols. In 8 to 10 animals in each experimental group, global low-flow ischemia was induced by reducing the coronary flow to 10%, resulting in a reduction of aortic pressure from 51 to 11 mm Hg. Low-flow ischemia was maintained for 30 min, followed by 30 min of reperfusion. External heart power (EHP) per gram of left ventricular (LV) wet weight was calculated: EHP_{LV} (mJ/g per min) = pressure − volume work/heart rate. Dimethyl sulfoxide (DMSO; 0.01%; Sigma) as vehicle or Ac-DEVD-CHO in 0.01% DMSO final (0.1 to 1 μmol/l; Alexis) was started 15 min before ischemia or 5 min before reperfusion and given throughout the reperfusion period.

Immunoblotting. Tissue was homogenized in lysis buffer (10 mmol/l tris-HCl, pH 8.0; 1% Triton X-100; 0.32 mol/l sucrose; 5 mmol/l EDTA; and 1 mmol/l phenylmethylsulfonylfluoride), and proteins were separated by sodium dodecyl sulfate/polyacrylamide gel electrophoresis (SDS/PAGE), transferred to PVDF membranes and immunoblotted, as described (15), with the following primary antibodies: anti-troponin I (clone C5), anti-mouse actin (both Chemicon) or anti-caspase-3 (p17 subunit, Santa Cruz Biotechnology). Bound antibodies were studied by chemiluminescence (Amersham).

Caspase-3 and calpain enzymatic assays. Myocardial tissue was homogenized as described previously, and 200 μg of protein was used in 700 μl of caspase-assay buffer (100 mmol/liter HEPES, pH 7.5; 0.32 mol/l sucrose; 100 mmol/l NaCl; 0.1% CHAPS; 2 mmol/l dithiotreitol; 10 μg/ml aprotinin; 10 μg/ml leupeptin; and 10 μg/ml pepstatin A), with 0.24 mmol/l of DEVD-AFC as the fluorogenic caspase-3 substrate, as described (16). For measuring calpain-like activity, 200 μg of protein was used in 600 μl of calpain-assay buffer (60 mmol/l imidazol, pH 7.5; 5 mmol/l L-cysteine; 0.2% Triton X-100; 5 mmol/l $CaCl_2$; 5 mmol/l dithiotreitol; 10 μg/ml aprotinin; 10 μg/ml leupeptin; and 10 μg/ml pepstatin A), with 0.24 mmol/l of fluorogenic calpain substrate I (Calbiochem). Purified calpain I (5 U; Calbiochem) was used as indicated, and after 60 to 100 s, calpain inhibitor I (Roche) or Ac-DEVD-CHO was added to the reaction mixture, while the increase in fluorescence was still linear.

Detection of apoptosis. The DNA strand breaks were analyzed in situ using 5-μm sections with terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-biotin nick end-labeling (TUNEL), as described (15). Internucleosomal DNA fragmentation ("DNA laddering") was detected by incubation of phenol-chloroform extracted DNA with 5 U of Klenow polymerase and 0.5 μCi of (α-^{32}P)-dCTP, followed by gel electrophoresis, as described (16).

Statistical analysis. All data are presented as the mean value ± SEM. Comparisons were performed by analysis of variance for comparison of multiple measurements or by the paired or unpaired Student *t* test. A Bonferroni correction for multiple comparisons was used to determine the level of significance. A p value <0.05 was considered statistically significant.

RESULTS

Ac-DEVD-CHO improves post-ischemic contractile recovery in a model of myocardial stunning of isolated working hearts of rats. As an experimental model of myocardial stunning, we used isolated working hearts of rats (17). Figure 1A illustrates the experimental protocol: after initial stabilization for 15 min, the hearts were treated with Ac-DEVD-CHO (0.1 to 1 μmol/l) or vehicle (0.01% DMSO) for 15 min before ischemia (8 to 10 hearts per experimental group). After 30 min of low-flow ischemia, reperfusion was established for 30 min. For the first 15 min of reperfusion, the hearts were perfused in the Langendorff mode to allow for recovery. Then, perfusion was switched back to the working-heart mode. Under these conditions,

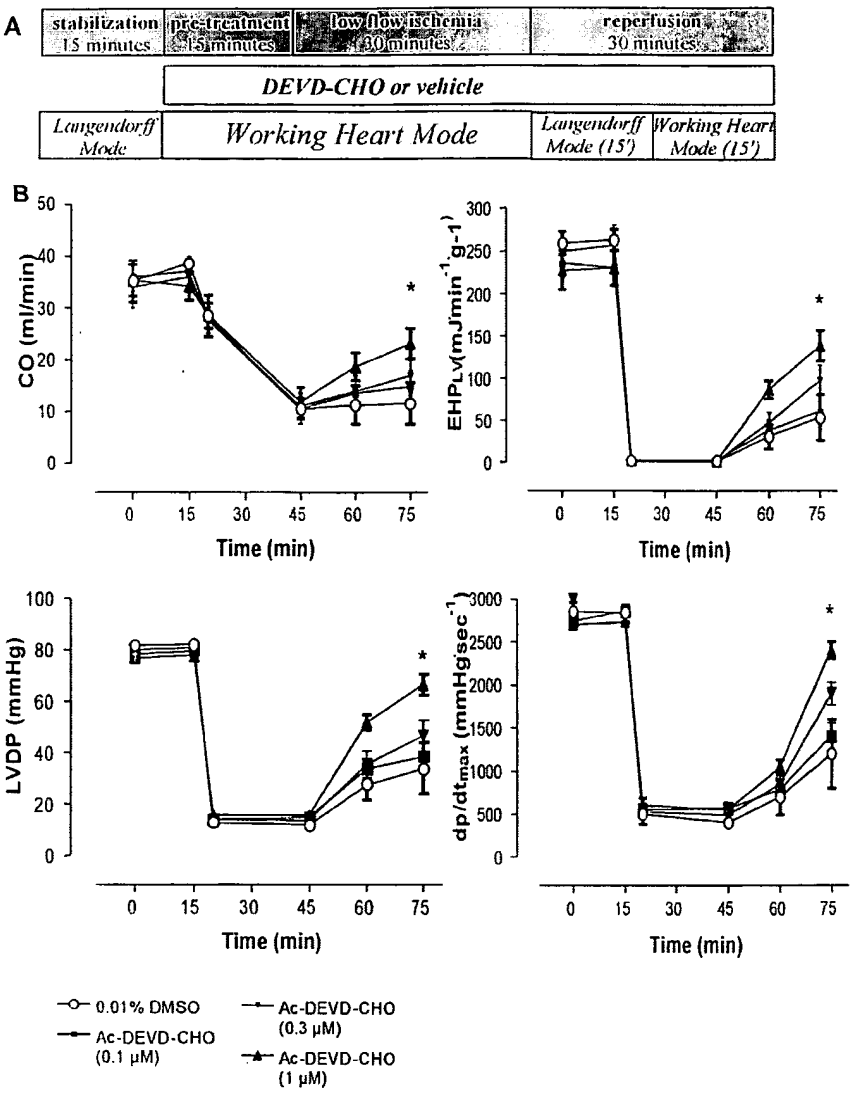

Figure 1. The caspase-3 inhibitor Ac-DEVD-CHO improves contractile recovery of isolated working hearts of rats exposed to ischemia and reperfusion. **(A)** Schematic diagram of the experimental set-up. **(B)** Effects of the caspase-3 inhibitor Ac-DEVD-CHO (0.1 to 1 μmol/liter) or vehicle (0.01% DMSO) on cardiac output (CO), external heart power per gram of LV wet weight (EHP_{LV}), left ventricular developing pressure (LVDP) and contractility (dp/dt_{max}). Data are presented as the mean value ± SEM; there are 8 to 10 animals per group. *$p < 0.05$ versus vehicle-treated group.

the residual 10% coronary flow during ischemia largely prevented MI (17).

In the control group, cardiac output, external heart power, (LV) developing pressure, myocardial contractility and aortic and coronary flow (data not shown) severely decreased during the ischemic period and remained depressed during reperfusion (Fig. 1B). Treatment of isolated hearts with 0.1 to 1 μmol/l of Ac-DEVD-CHO (starting 15 min before ischemia) did not affect cardiac performance at baseline or during ischemia. However, Ac-DEVD-CHO dose-dependently and significantly ($p < 0.05$ for 1 μmol/l) improved the contractile recovery of post-ischemic myocardium. Just 1 μmol/l of Ac-DEVD-CHO significantly enhanced cardiac output, external heart power, LV developing pressure, myocardial contractility (Fig. 1B) and aortic and coronary flow (data not shown).

Figure 2 shows that only 20% to 45% (calculated values relative to pre-ischemic baseline value) of post-ischemic recovery of the various variables was observed in the vehicle-treated group. This indicates severe cardiac dysfunction, even after restoration of perfusion. Treatment with 1 μmol/l of Ac-DEVD-CHO increased the relative recovery of all variables to 60% to 85% of the pre-ischemic values (Fig. 2).

Ac-DEVD-CHO is equally effective when administered before reperfusion. To investigate whether Ac-DEVD-CHO protects the myocardium from stunning when administered after the onset of ischemia, Ac-DEVD-CHO (1 μmol/l) was started 5 min before reperfusion. This scenario more closely resembles the common clinical situation of patients presenting with acute coronary syndromes.

As shown in Figure 2, the group treated with 1 μmol/liter of Ac-DEVD-CHO, started 5 min before reperfusion, led

JACC Vol. 38, No. 7, 2001
December 2001:2063–70

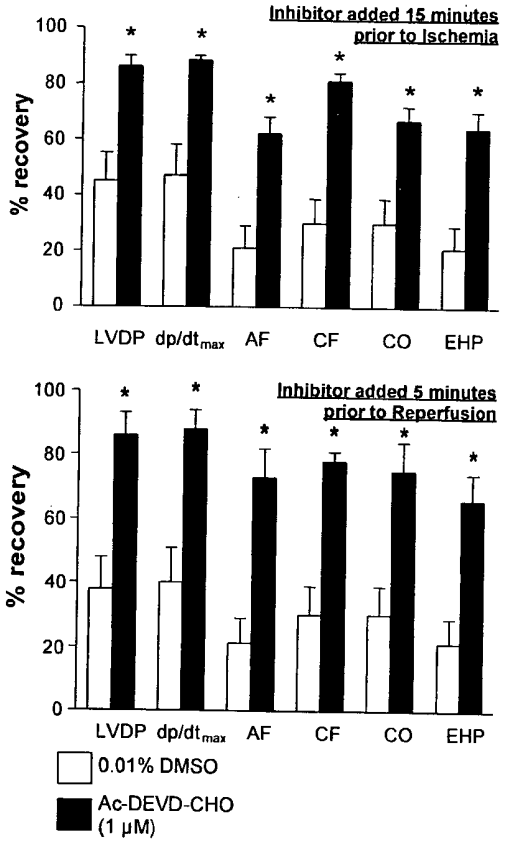

Figure 2. Ac-DEVD-CHO is effective when given 5 min before reperfusion. The effect of Ac-DEVD-CHO on contractile recovery is shown, calculated as percent recovery compared with baseline (pre-ischemic) values of left ventricular developing pressure (LVDP), contractility (dp/dt_{max}), aortic flow (AF), coronary flow (CF), cardiac output (CO) and external heart power (EHP). Data are presented as the mean value ± SEM; there are 8 to 10 animals in each group. Ac-DEVD-CHO was started 15 min before ischemia **(top)** or 5 min before reperfusion **(bottom)**. Note the similar beneficial effect of Ac-DEVD-CHO on the relative recovery. *$p < 0.05$ versus vehicle-treated group. **Open bars** = 0.01% DMSO; **solid bars** = 1 μmol/l of Ac-DEVD-CHO.

to a significantly ($p < 0.05$) improved post-ischemic recovery of cardiac performance, compared with the vehicle-treated group. Specifically, Ac-DEVD-CHO started 5 min before reperfusion improved cardiac output, external heart power, LV developing pressure, myocardial contractility and aortic and coronary flow to a similar degree, compared with Ac-DEVD-CHO started 15 min before ischemia (Fig. 2).

Ac-DEVD-CHO blocks ischemia/reperfusion-induced activation of caspase-3. Next, we investigated the effects of ischemia/reperfusion and Ac-DEVD-CHO on the activation of caspase-3. Caspase-3 exists as an inactive zymogen of 32 kD and is activated by proteolytic processing into p17 and p12 subunits (11,12).

Immunoblotting of rat heart extracts for the p17 subunit of caspase-3 (Fig. 3A, left) detected only the inactive 32-kD zymogen in nonischemic hearts. Ischemia/reperfusion caused caspase-3 activation, with the appearance of the processed p17 subunit in the vehicle group. Treatment with Ac-DEVD-CHO before reperfusion almost completely blocked activation of caspase-3 induced by ischemia/reperfusion.

To determine catalytic activity of caspase-3 in rat heart extracts, we used a fluorogenic caspase-3 substrate (Fig. 3A, right). When compared with the control group, caspase-3–like activity was more than fivefold increased by ischemia/reperfusion in the vehicle-treated group ($p < 0.05$). Treatment with Ac-DEVD-CHO from 5 min before reperfusion abolished activation of caspase-3, with proteolytic activity remaining close to baseline levels ($p < 0.05$ vs. vehicle-treated group).

These results demonstrate that Ac-DEVD-CHO is able to block the ischemia/reperfusion-induced activation of caspase-3.

Effects of Ac-DEVD-CHO on apoptosis. Increased apoptosis has been implicated in MI (9,10). Because Ac-DEVD-CHO can block apoptosis in various cell types (12), we determined the rate of apoptotic cells by TUNEL staining and internucleosomal DNA fragmentation.

As shown in Figure 3B, we detected single TUNEL-positive cells in the nonischemic myocardium, as well as in the vehicle-treated and Ac-DEVD-CHO–treated myocardium subjected to ischemia/reperfusion. The TUNEL-positive cells appeared to be mostly cardiomyocytes. Quantitative analysis (5 sectors of ~500 cells/sector; n = 4 hearts in each group) revealed a significant ($p < 0.05$) increase in the number of TUNEL-positive cells in both the vehicle-treated (0.38 ± 0.18%) and Ac-DEVD-CHO–treated (0.42 ± 0.31%) groups, compared with nonischemic control group (0.05 ± 0.03%).

This increase in the rate of myocardial apoptosis during ischemia/reperfusion was confirmed by the internucleosomal DNA fragmentation assay. Compared with the nonischemic control group, ischemia/reperfusion caused an increase in DNA strand breaks, with the typical "ladder" pattern (Fig. 3B, right). The ischemia/reperfusion-induced increase in apoptosis was similar in the vehicle-treated and Ac-DEVD-CHO–treated groups.

Effects of Ac-DEVD-CHO on troponin I degradation and calpain activity. Because the beneficial effect of Ac-DEVD-CHO on contractile recovery was largely independent of apoptosis, we investigated whether Ac-DEVD-CHO may inhibit other cysteine proteases, such as calpain. Because calpain-mediated cleavage of the thin-filament regulatory protein troponin I has been suggested as a molecular mechanism of stunning (1), we investigated the effects of Ac-DEVD-CHO on troponin I degradation and calpain activity.

Immunoblotting with an anti–troponin I antibody showed increased appearance of the characteristic troponin I fragment in vehicle-treated and Ac-DEVD-CHO–treated hearts subjected to ischemia/reperfusion (Fig. 4A). However, Ac-DEVD-CHO started 5 min before reperfusion did not quantitatively alter the troponin I degradation.

Using a fluorogenic calpain substrate, we were unable to

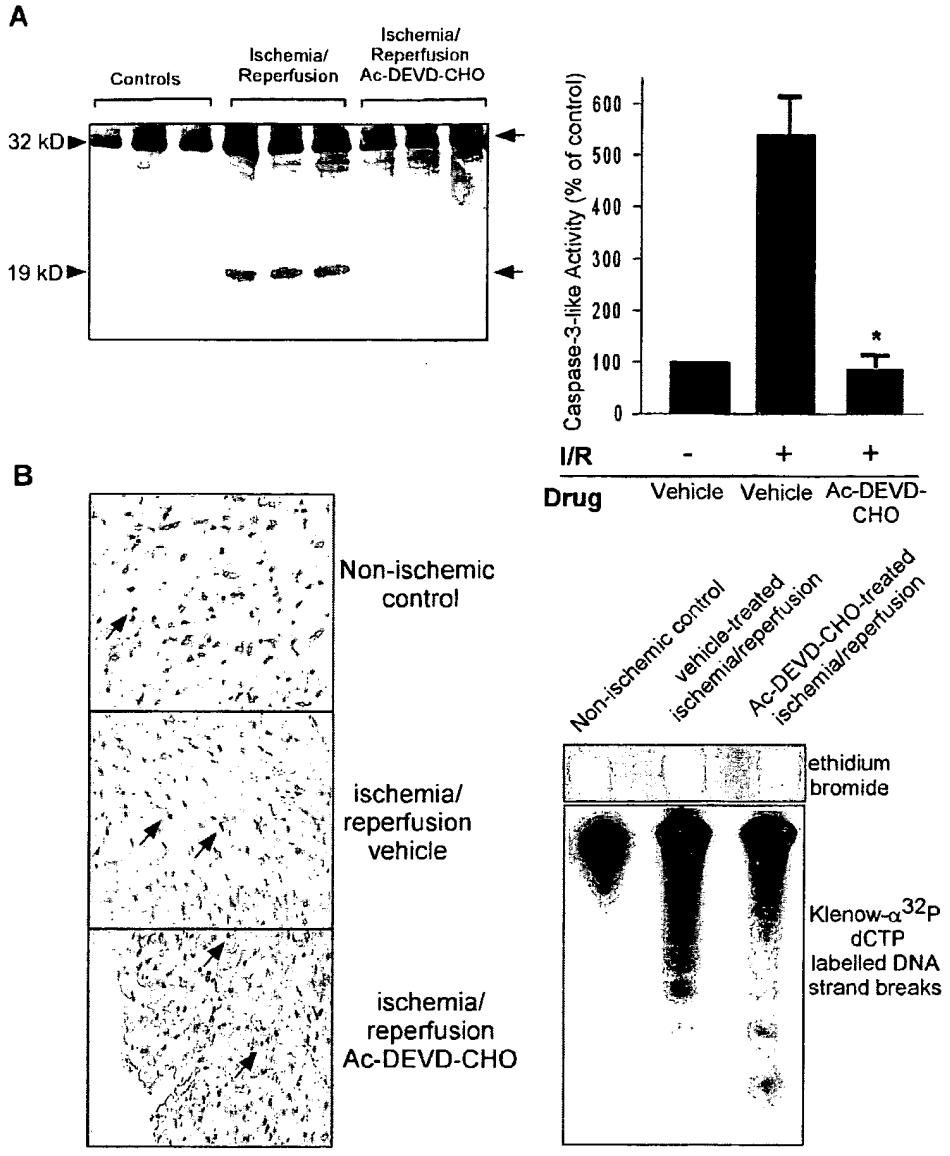

Figure 3. Ac-DEVD-CHO blocks ischemia/reperfusion (I/R)-induced caspase-3 activation but not apoptosis. **(A, left),** Immunoblotting of rat hearts with an antibody against the p17 subunit of caspase-3; three independent experiments are shown. **Arrowheads** indicate the molecular weight of the inactive zymogen (32 kD) and the processed, active subunit (17 kD). **(A, right),** Caspase-3–like proteolytic activity of rat hearts (percent increase relative to the proteolytic activity in control hearts), as measured with a fluorogenic substrate of caspase-3. *$p < 0.05$. **(B, left),** Representative photomicrographs of myocardial sections stained with the TUNEL technique to detect apoptotic cells in situ in a nonischemic control heart **(top),** a vehicle-treated heart subjected to ischemia/reperfusion **(middle)** or an Ac-DEVD-CHO-treated heart subjected to ischemia/reperfusion **(bottom).** **Arrows** indicate TUNEL-positive cells. Magnification 200×. **(B, right)** Autoradiogram of radioactively labeled DNA strand breaks, followed by agarose gel electrophoresis; results from four independent samples in each group. Equal loading was verified by ethidium bromide staining. Note the internucleosomal DNA fragmentation ("ladder pattern") induced by ischemia/reperfusion.

reproducibly detect measurable calpain-like proteolytic activity in any of the rat hearts (data not shown). To address a potential nonspecific effect of Ac-DEVD-CHO on calpain, we determined the effects of Ac-DEVD-CHO on purified calpain I in vitro. The addition of calpain to its substrate caused a linear increase in fluorescence (Fig. 4B). While the increase was still linear, calpain inhibitor I or Ac-DEVD-CHO was added. Although 100 nmol/l of calpain inhibitor I almost completely inhibited calpain I, the same concentration of Ac-DEVD-CHO had little inhibitory effect. Quantitative evaluation of the remaining catalytic activity after addition of the inhibitor (Fig. 4C) showed that the cysteine protease calpain could be principally inhibited by the aldehyde Ac-DEVD-CHO at higher concentrations (≥ 1 μmol/l). However, 10-fold higher concentrations of Ac-DEVD-CHO were needed to achieve an equal inhibition of calpain, compared with calpain inhibitor I. Taken together, these results indicate that, in vivo, Ac-DEVD-CHO does not act primarily through calpain inhibition.

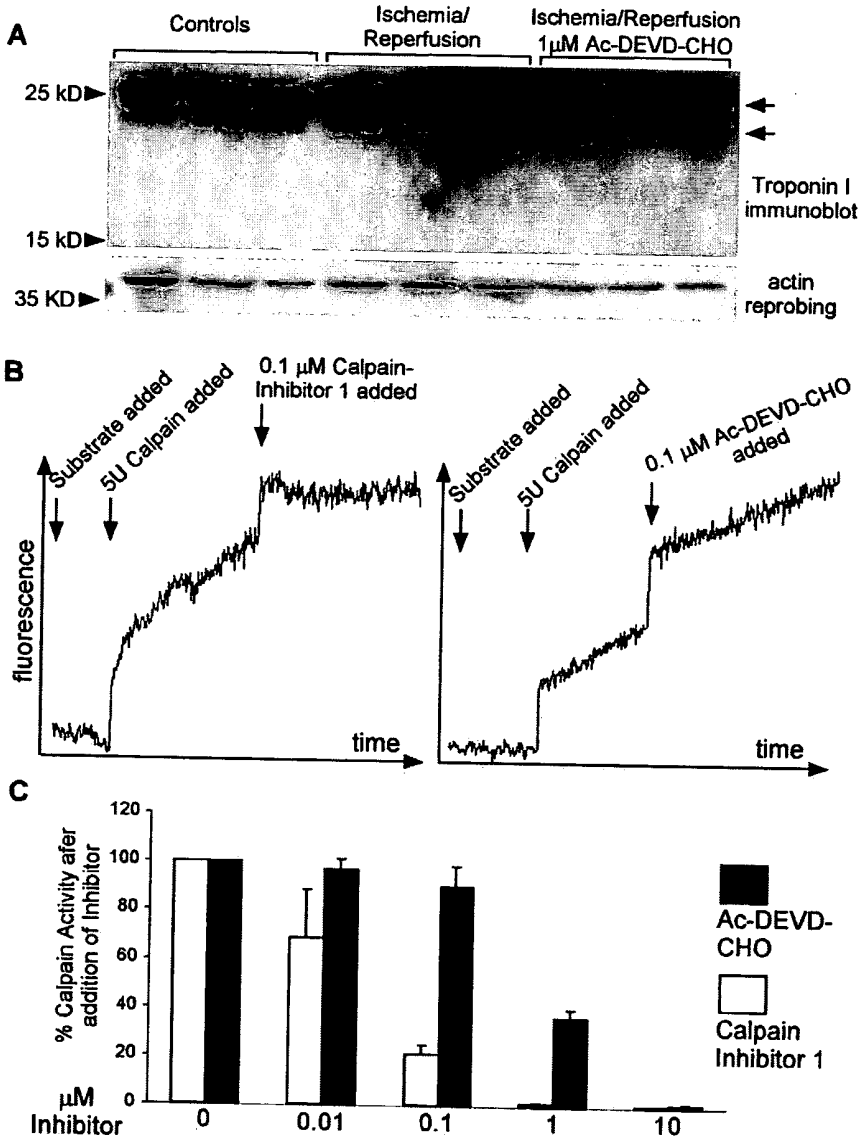

Figure 4. Effects of DEVD-CHO on troponin I degradation and calpain activity. **(A)** Immunoblots of rat hearts with an antibody against troponin I **(upper blots)** or actin **(lower blots)**. **Arrowheads** indicate the molecular weight. The **top right arrow** indicates full-length troponin I; the **lower arrow** indicates the major degradation product. **(B)** Effects of 0.1 μmol/l of calpain inhibitor I **(left)** or Ac-DEVD-CHO **(right)** on the catalytic activity of 5 U of calpain I, as measured in vitro with a fluorogenic calpain I substrate. **(C)** Quantitative evaluation of the percent activity after addition of the inhibitor, compared with the activity measured before addition of the inhibitor. Data are presented as the mean value ± SEM from three independent measurements for each data point.

DISCUSSION

The caspase-3 inhibitor Ac-DEVD-CHO significantly improves post-ischemic contractile recovery of isolated working hearts of rats. Interestingly, Ac-DEVD-CHO was effective even when given after the onset of low-flow ischemia. The effects of caspase-3 inhibition appeared to be largely independent of cardiomyocyte apoptosis.

Myocardial stunning and caspase-3 activation. The role of apoptosis in the pathogenesis of myocardial stunning is unknown. Because caspase-3 is a key protease that executes apoptosis, we investigated whether caspase-3 inhibition reduces myocardial stunning in an isolated working-heart rat model. This model is well established and allows study of myocardial contractility in the intact heart, independent of compounding factors, such as sympathetic activity and activation of an immune response (17). Indeed, caspase-3 was activated in our model of myocardial stunning. Thus, our data confirm and extend two recently published studies demonstrating activation of caspase-3 in a rat and rabbit model of MI (9,10). As hypothesized, Ac-DEVD-CHO was able to block the

ischemia/reperfusion-induced activation of caspase-3 in our experimental model.

Ac-DEVD-CHO functionally improves stunned myocardium. In contrast to the vehicle, the caspase-3 inhibitor Ac-DEVD-CHO dose-dependently and significantly improved post-ischemic contractile recovery. Just 1 μmol/l led to almost a doubling of all contractile variables to 60% to 85% of the pre-ischemic values. These good recovery rates principally achievable with an appropriate pharmacologic intervention also demonstrate that low-flow ischemia/reperfusion in our model causes mainly myocardial stunning but little if any MI. Importantly, Ac-DEVD-CHO was equally potent in reducing myocardial stunning when given 5 min before reperfusion, instead of before the induction of ischemia. This scenario being given resembles the clinical situation.

Myocardial stunning and apoptosis. Although the beneficial effect of caspase inhibitors in experimental MI has been attributed to a reduced rate of apoptosis (7,10), our data do not support a role for the inhibition of apoptosis as the mechanism by which Ac-DEVD-CHO acts on the contractile recovery of stunned myocardium. Ac-DEVD-CHO neither reduced the number of TUNEL-positive cells, nor the amount of internucleosomal DNA fragmentation. Although ischemia/reperfusion-induced caspase-3-activation was associated with a minor increase in the number of apoptotic cells and fragmented DNA, there was no significant effect of DEVD-CHO on the degree of apoptosis. Thus, apoptosis of cardiac myocytes in this experimental setting seems to be mediated by a caspase-independent pathway. Indeed, a recent study suggests that other signalling pathways can induce apoptosis independent of the caspase cascade (18). Moreover, it remains to be clarified whether an increase of apoptosis of ~0.3% has any significant outcome with regard to the early functional integrity of the heart. Therefore, the profound improvement in post-ischemic contractile recovery by the caspase-3 inhibitor Ac-DEVD-CHO appears to be independent of apoptosis in the experimental setting used. These findings indicate that the integrity of the contractile apparatus may be adversely affected by caspase-3 activation in the myocardium during stunning.

Degradation of contractile proteins during myocardial stunning. Selective troponin I degradation has been reported in some (1–5,19), but not all (20), models of myocardial stunning. Transgenic overexpression of the major proteolytic troponin I product in the heart is sufficient to recapitulate many aspects of myocardial stunning (21). Subsequent studies suggested calpain-mediated cleavage of troponin I as an underlying mechanism (2–5,19), and calpain inhibitor I is able to reduce infarct size (22) and improve myocardial stunning (23). Because Ac-DEVD-CHO may inhibit other cysteine proteases, such as calpain, we investigated whether Ac-DEVD-CHO may inhibit troponin I degradation. Consistent with published reports (2–5,19), ischemia/reperfusion in isolated working hearts of rats resulted in partial troponin I degradation, which was not quantitatively affected by Ac-DEVD-CHO. In vitro, purified calpain could be inhibited by Ac-DEVD-CHO. However, 1 μmol/l of Ac-DEVD-CHO achieved only partial calpain inhibition, indicating that it acts in vivo, primarily through pathway(s) other than calpain inhibition. However, we cannot exclude that Ac-DEVD-CHO exerts some of its beneficial effects by partial calpain inhibition. Calpain or other proteinase(s) may play a pathogenic role in our model, because we achieved only incomplete recovery with Ac-DEVD-CHO.

Alternatively, Ac-DEVD-CHO may inhibit cleavage of other contractile proteins. Through a data bank search with caspase cleavage motifs, we identified a putative caspase cleavage site ($DEVD^{63}$) in cardiac troponin C (12). However, immunoblots of rat hearts with troponin C antibodies did not show any troponin C degradation during ischemia-reperfusion (data not shown). Further experiments will be required to identify the molecular target(s) of caspase activation during stunning.

Conclusions. Taken together, in an isolated working-heart rat model of myocardial stunning, inhibition of ischemia/reperfusion-induced caspase-3 activation by Ac-DEVD-CHO results in a substantial improvement of post-ischemic contractile recovery. The observed effects appear to be independent of suppression of apoptosis, but most likely involve both caspase and calpain inhibition. Regardless of the underlying mechanism(s), the use of a caspase-3 inhibitor represents a potentially clinically relevant, novel therapeutic strategy to reduce myocardial stunning.

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EVIDENCE OF APOPTOSIS IN ARRHYTHMOGENIC RIGHT VENTRICULAR DYSPLASIA

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ABSTRACT

Background Arrhythmogenic right ventricular dysplasia, a disorder that may lead to severe ventricular arrhythmias and sudden death, is characterized by the progressive replacement of myocardial cells by fat and fibrous tissue. We examined whether the loss of myocardial cells in this disease could result from cell death by apoptosis (programmed cell death).

Methods Specimens obtained at autopsy from the right ventricular myocardium of eight patients with arrhythmogenic right ventricular dysplasia and four age-matched normal subjects were analyzed. To identify individual cells undergoing apoptosis, we performed in situ end-labeling of fragmented DNA on paraffin sections using biotinylated deoxyuridine triphosphate and the enzyme terminal deoxynucleotidyl transferase. We also examined the level of expression of CPP-32, a cysteine protease required for apoptotic cell death in mammalian cells, using immunohistochemical techniques.

Results Apoptosis was detected in the right ventricular myocardium of six of the eight patients with arrhythmogenic right ventricular dysplasia and was absent in the controls. High levels of expression of CPP-32 were associated with positive in situ end-labeling of fragmented DNA.

Conclusions These results indicate that apoptotic myocardial cell death occurs in arrhythmogenic right ventricular dysplasia and may contribute to the loss of myocardial cells in this disorder. (N Engl J Med 1996;335:1190-6.)

APOPTOSIS, or programmed cell death, is a highly regulated and active process that contributes to the control of cell number during development and to the maintenance of many adult tissues.[1-3] It is triggered by the activation of an internally encoded suicide program as a result of either extrinsic or intrinsic signals.[4] Apoptosis differs morphologically from necrosis: it is characterized by blebbing of the cell membrane, a reduction in cell volume, condensation of nuclear chromatin, and endonucleolytic degradation of DNA at nucleosomal intervals.[2] Apoptotic bodies are digested or phagocytosed by adjacent cells or macrophages without inducing an inflammatory response.

The crucial role of apoptosis in pathologic conditions is increasingly being recognized.[5,6] Recently, apoptosis was reported as a possible mechanism for the loss of myocardial cells in an infant with Uhl's anomaly.[7] Arrhythmogenic right ventricular dysplasia, a form of right ventricular cardiomyopathy that commonly leads to severe ventricular arrhythmias and sudden death,[8,9] is characterized by noninflammatory loss of myocardial cells and their progressive replacement by fat and fibrous tissue.[8-11] We hypothesized that this loss of myocardial cells in arrhythmogenic right ventricular dysplasia may result from cell death by apoptosis.[6]

METHODS

Cardiac Specimens

Sections from the right ventricle of eight patients (five men and three women; mean [±SD] age, 47±15 years) who died of arrhythmogenic right ventricular dysplasia were examined. Five of the patients had documented ventricular arrhythmias. The final diagnosis of right ventricular dysplasia was based on the following established criteria[9-12]: massive infiltration of the right ventricular wall by fat tissue, with surviving strands of cardiomyocytes embedded in or bordered by fibrous tissue (Fig. 1), a finding typically distinct from the patchy replacement of myocardium by fat and fibrous tissue that may result from chronic myocarditis and also distinct from the strands of cardiomyocytes found in fatty tissue without fibrosis, which could be a normal variant[10]; sparing of subendocardial myocardium which may show trabecular hypertrophy or disarrangement (Fig. 1); substantial sparing of the left ventricular myocardium; and the absence of other cardiac diseases. These criteria were present in all the patients studied. Extensive mononuclear infiltrates and diffuse interstitial fibrosis superimposed on the typical pattern of arrhythmogenic right ventricular dysplasia were observed on histologic analysis in only one patient.

Sections from the right ventricle of four normal subjects (three men and one woman; mean age, 41±16 years) who died of other, noncardiac causes served as controls. None of these control subjects met any of the histologic criteria for right ventricular dysplasia. Although the interval between death and autopsy did not affect the detection of apoptosis, only cases in which this interval was 24 hours or less were included. Tissues were fixed in 10 percent buffered formalin and embedded in paraffin. Four to six sections (6 μm thick) from each paraffin block were analyzed for the presence of apoptosis.[13]

In Situ Detection of Apoptotic Cells

Sections were deparaffinized, transferred to xylene, and rehydrated in descending concentrations of alcohol (100 percent,

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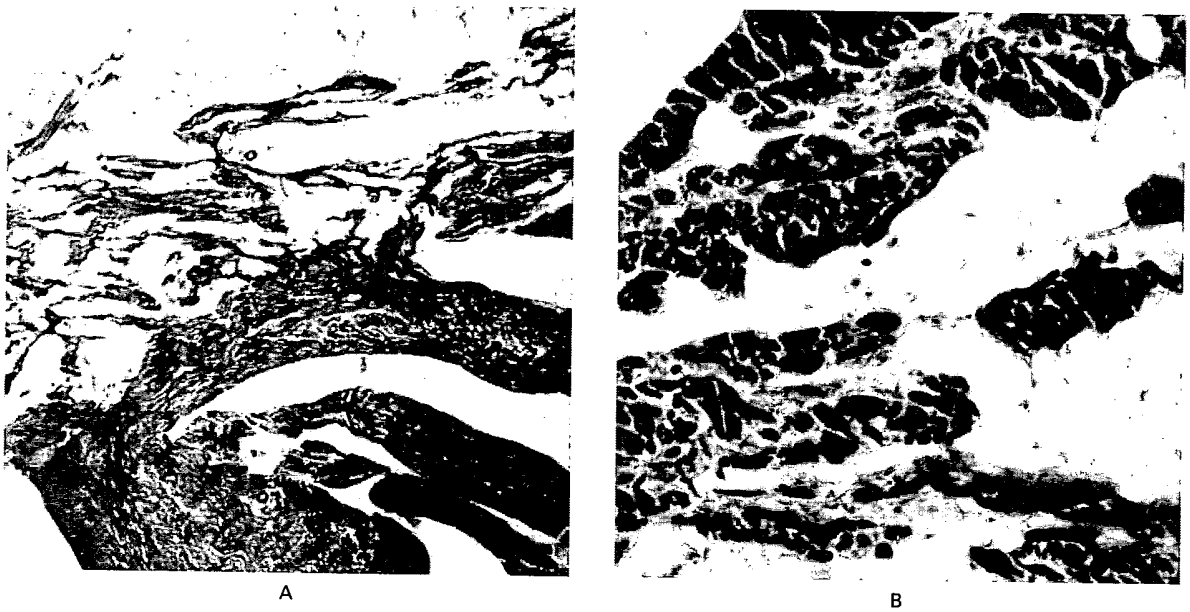

A B

Figure 1. Free Wall of the Right Ventricle of a Patient with Right Ventricular Dysplasia.

In Panel A, there is a large amount of adipose tissue occupying the mediomural and subepicardial layers (hematoxylin–phloxin–safran staining, ×10). In Panel B, high magnification reveals surviving strands of myocardium bordered by or embedded in fibrous tissue. The presence of fibrous tissue is necessary for the diagnosis of right ventricular dysplasia[10] (hematoxylin–phloxin–safran staining, ×100).

95 percent, 70 percent, 50 percent, and 0 percent). After rehydration, the slides were incubated with 20 μg of proteinase K per milliliter in phosphate-buffered saline. Endogenous peroxidase was inactivated by 3 percent hydrogen peroxide. Tissue sections were stained with an ApopDETEK system (Enzo Diagnostics, Farmingdale, N.Y.) that identifies cells with internucleosomal fragmentation of DNA (apoptosis). The procedure was performed according to the manufacturer's instructions. The method is based on the preferential binding of terminal deoxynucleotidyl transferase (TdT) to the 3′-hydroxyl ends of DNA.[13] Briefly, residues of biotinylated deoxyuridine triphosphate (dUTP) were catalytically added to the ends of DNA fragments with the enzyme TdT. For negative controls, deionized water was used instead of TdT. After end-labeling, the sections were incubated with avidin–horseradish peroxidase and stained with diaminobenzidine to detect the biotin-labeled nuclei. Apoptotic bodies stained brown. Positive controls consisted of rat mammary glands obtained on the fourth day after weaning (Oncor, Gaithersburg, Md.). Four to six sections from each specimen were examined. Sections were first examined under light microscopy at low magnification (×100), allowing estimation of the percentage of surface area occupied by apoptotic cells. Then, 10 random fields per section from the regions with apoptotic cells were examined at a higher magnification (×400) to calculate the percentage of myocardial nuclei with DNA fragmentation. Cardiomyocytes, which were well-shaped, elongated, and striated cells, were easily distinguished morphologically from other rare nonmyocytes under a light microscope at high magnification. In addition to the in situ end-labeling technique, adjacent sections stained with hematoxylin and eosin were examined for signs of apoptosis.[14] The pathologist analyzing the specimens was unaware of the diagnosis in 9 of the 12 cases examined.

Immunohistochemical Detection of Protease CPP-32

CPP-32 is a cysteine protease required for the initiation of apoptotic cell death.[15] It is related to interleukin-1β–converting enzyme (ICE) and CED-3, the product of a gene required for programmed cell death in the nematode *Caenorhabditis elegans*. CPP-32 is the specific ICE/CED-3–like mammalian cysteine protease that cleaves and inactivates poly(adenosine diphosphate ribose) polymerase, an enzyme involved in DNA repair and genome integrity, and thus may be the human equivalent of CED-3.[15] Therefore, to provide further evidence of the occurrence of apoptosis in arrhythmogenic right ventricular dysplasia, we analyzed the level of expression of CPP-32 in the right ventricles of the patients and controls using immunohistochemical techniques.

After deparaffinization and rehydration, the sections were incubated with 1:10 normal horse serum for 30 minutes at room temperature, washed once in phosphate-buffered saline, and stained with a mouse monoclonal anti–CPP-32 antibody (Transduction Laboratories, Lexington, Ky.) at a dilution of 1:1000. The slides were washed in phosphate-buffered saline and then incubated with biotinylated horse antimouse IgG (Vector Laboratories, Burlingame, Calif.) at a dilution of 1:200. Stains were visualized with an avidin–alkaline phosphatase–substrate system (Vectastain ABC Kit and Vector Red, Vector Laboratories, Burlingame, Calif.). As a negative control, serial sections were stained without the primary antibody against CPP-32.

RESULTS

Evidence of Apoptosis

In situ end-labeling of fragmented DNA with TdT and biotinylated dUTP did not reveal apoptosis in sections of right ventricular myocardium from the four normal adult subjects (Fig. 2A). In contrast, sections of right ventricular myocardium from six of the eight patients with arrhythmogenic right ventricular dysplasia showed numerous cells with genomic DNA fragments in their nuclei (Fig. 2B, 2C,

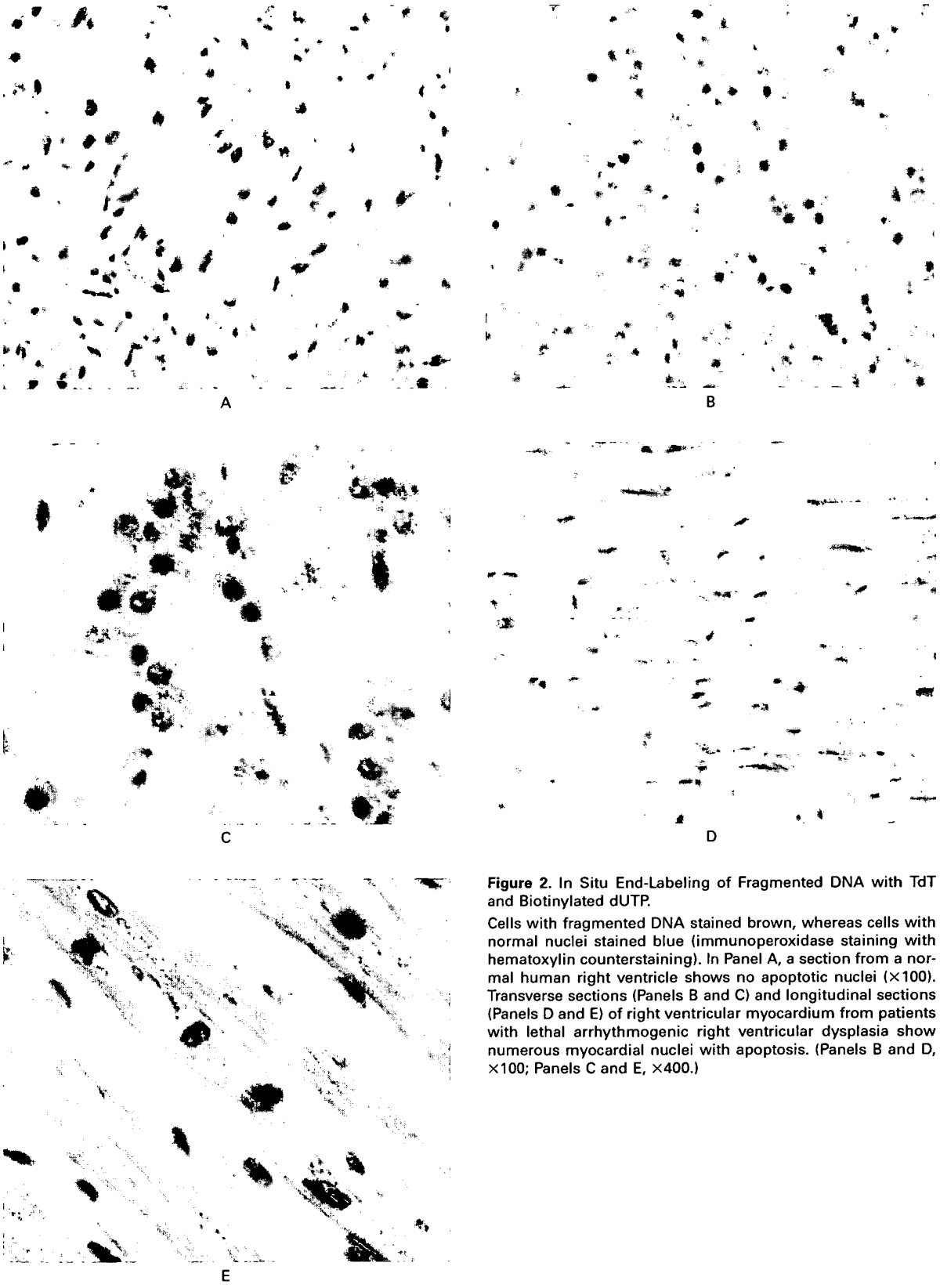

Figure 2. In Situ End-Labeling of Fragmented DNA with TdT and Biotinylated dUTP.

Cells with fragmented DNA stained brown, whereas cells with normal nuclei stained blue (immunoperoxidase staining with hematoxylin counterstaining). In Panel A, a section from a normal human right ventricle shows no apoptotic nuclei (×100). Transverse sections (Panels B and C) and longitudinal sections (Panels D and E) of right ventricular myocardium from patients with lethal arrhythmogenic right ventricular dysplasia show numerous myocardial nuclei with apoptosis. (Panels B and D, ×100; Panels C and E, ×400.)

2D, and 2E). The majority of these cells were easily recognized as myocardial cells under a light microscope at high magnification, since they were well shaped, elongated, and striated (Fig. 2C and 2E). The apoptotic myocardial cells were frequently in regions of myocardium not already invaded by adipocytes and fibrosis. They were less frequently in regions replaced by fat and fibrous tissue, where rare, nonapoptotic cardiomyocytes were still present. Both the extent of regions with apoptotic cells and the percentage of apoptotic myocardial cells in these regions varied among the patients (Table 1). An inflammatory reaction was detected in sections from one of the eight patients (Patient 1). This patient had the highest percentage of apoptotic myocardial cells, and some inflammatory cells were also apoptotic (Table 1 and Fig. 3).

The detection of apoptotic cells by in situ end-labeling of fragmented DNA was supported by the fact that pathological criteria for apoptosis were also met. In adjacent sections stained with hematoxylin and eosin, nuclei of numerous myocardial cells showed marginated masses of chromatin along with discrete, well-preserved apoptotic bodies (Fig. 4), typical pathological features of apoptosis.[14] No sign of apoptosis was seen in adjacent sections stained with hematoxylin and eosin from the four normal subjects (data not shown).

Expression of CPP-32 in Right Ventricular Myocardium from Patients with Arrhythmogenic Right Ventricular Dysplasia

Protease CPP-32 is important for the induction of apoptotic cell death in mammalian cells.[15] It was undetectable or barely detectable in the right ventricles of the four normal subjects (Fig. 5A), as well as in the two patients with no evidence of apoptosis. However, cardiomyocytes from the right ventricles of the six patients with apoptosis showed high levels of immunoreactive CPP-32 (Fig. 5B). No staining was detected after omission of the primary anti–CPP-32 antibody.

DISCUSSION

We report the occurrence of apoptotic myocardial cell death in right ventricular dysplasia. James[6] has previously suggested that apoptosis may be a mechanism of cell death in arrhythmogenic right ventricular dysplasia.

In the present study, apoptosis was identified by in situ end-labeling of fragmented DNA with TdT and biotinylated dUTP, a commonly accepted method for the detection of the apoptotic process.[16] There has been concern about the ability of such in situ labeling methods to distinguish between cell necrosis and apoptosis. However, in this study the detection of a positive reaction with in situ end-labeling was correlated with the presence of typical

TABLE 1. EXTENT OF AREAS OF APOPTOSIS AND THE PERCENTAGE OF APOPTOTIC MYOCARDIAL NUCLEI IN THESE AREAS IN SECTIONS OF RIGHT VENTRICULAR MYOCARDIUM FROM PATIENTS WITH LETHAL ARRHYTHMOGENIC RIGHT VENTRICULAR DYSPLASIA.*

PATIENT NO.	EXTENT OF AREAS WITH APOPTOTIC NUCLEI	PROPORTION OF APOPTOTIC NUCLEI IN POSITIVE AREAS
	% of section	%
1	50	28
2	20	15
3	10	18
4	20	20
5	0	0†
6	15	22
7	15	14
8	0	0†

*Four to six sections from each specimen were examined. After in situ end-labeling of fragmented DNA, sections were examined under light microscopy at low magnification (×100), allowing an estimation of the surface area occupied by apoptotic cells. Then, 10 random fields per section from these positive areas were examined at high magnification (×400) to calculate the percentage of myocardial nuclei with DNA fragmentation.

†Only one apoptotic cell per section was found.

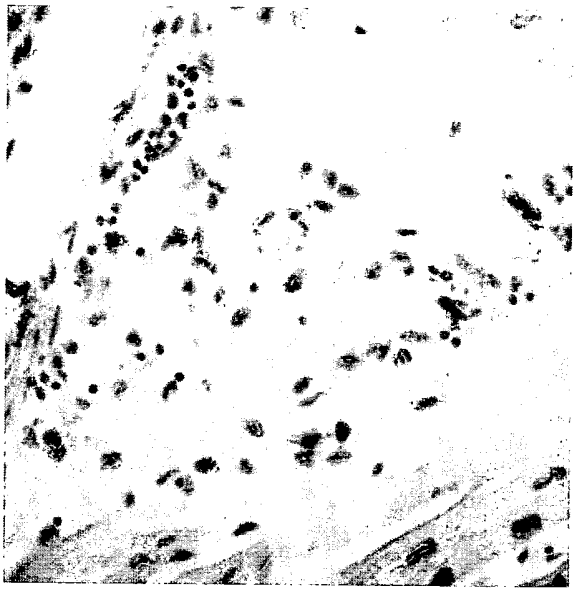

Figure 3. Apoptotic Nuclei in Nonmyocytes in an Inflammatory Reaction in One Patient (Immunoperoxidase Staining with Hematoxylin Counterstaining, ×250).

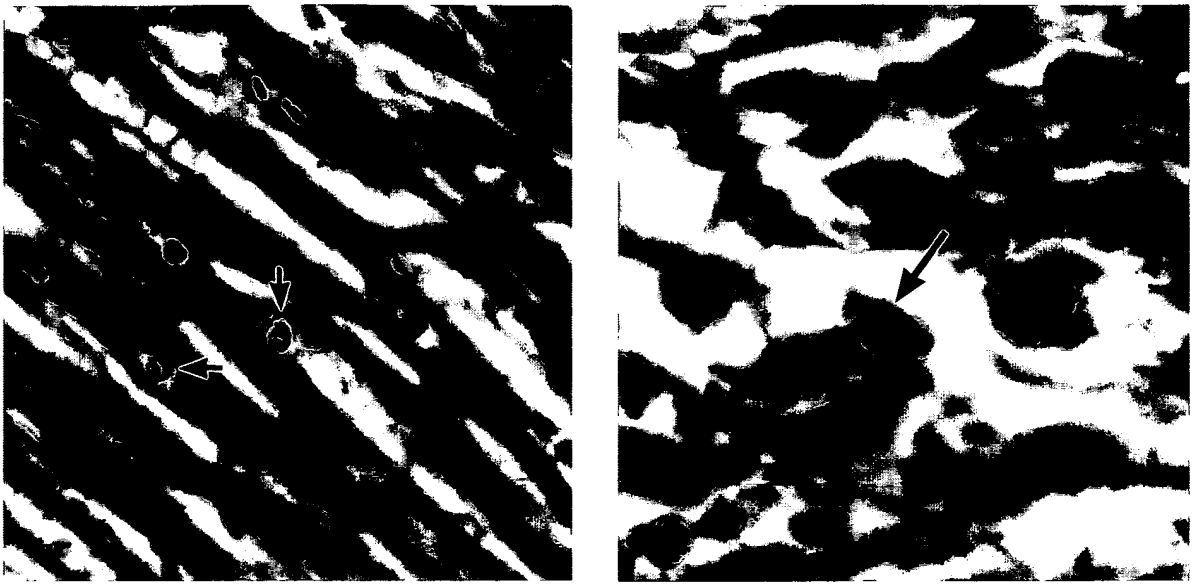

Figure 4. Sections of Right Ventricular Myocardium from Patients with Lethal Arrhythmogenic Right Ventricular Dysplasia (Hematoxylin and Eosin).

In Panel A, there are marginated masses of chromatin within myocardial nuclei in a longitudinal section (arrows) (×250). In Panel B there are multiple round, hyperdense nuclear fragments, whose appearance is consistent with that of apoptotic bodies, in a transverse section (arrow) (×400).

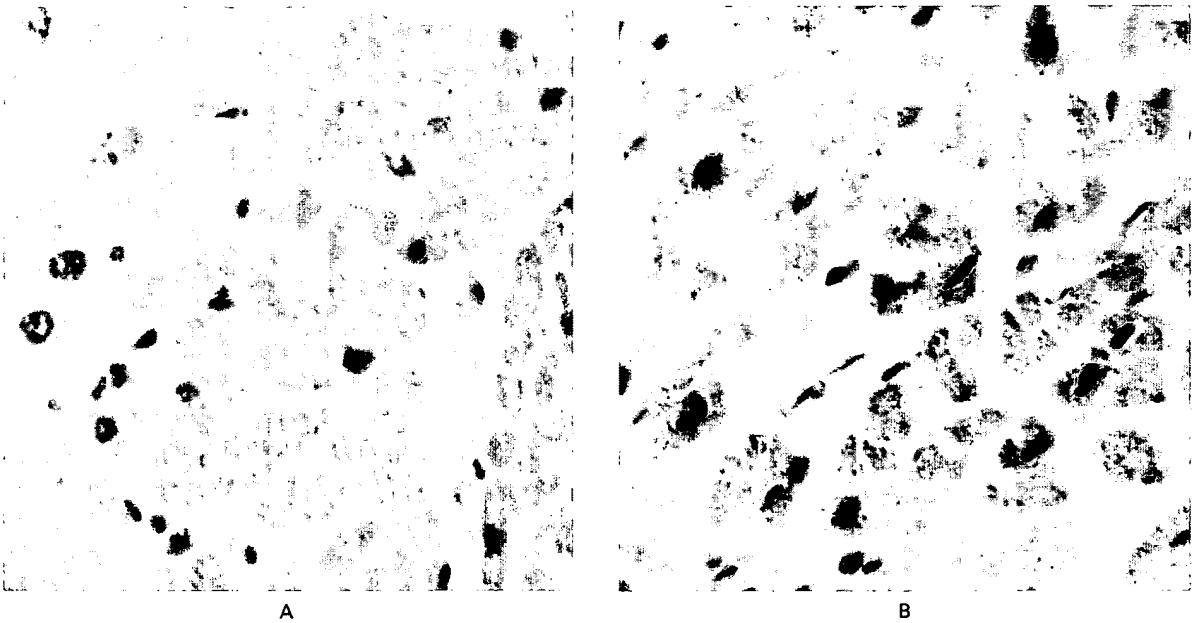

Figure 5. Immunohistochemical Detection of CPP-32.

In Panel A, normal right ventricular myocardium does not stain for CPP-32 (×400). In Panel B, right ventricular myocardium from a patient with right ventricular dysplasia stains intensely for CPP-32 (×400). The antibody against CPP-32 was detected with anti-mouse IgG conjugated with biotin and an avidin–alkaline phosphatase–substrate system in which positive cells stain red.

signs of apoptosis (marginated masses of chromatin and well-preserved apoptotic bodies) in sections stained with hematoxylin and eosin.[14] Moreover, the absence of an inflammatory reaction in most of our patients argues against necrosis and for apoptosis. Furthermore, no positive staining was detected in cardiomyocytes from age-matched normal subjects whose hearts were processed in the same manner as those of our patients, and the in situ end-labeling of fragmented DNA was not detected when the enzyme TdT was omitted.

To gain further evidence of apoptosis in arrhythmogenic right ventricular dysplasia and extend our findings, we examined the level of expression of protease CPP-32, whose activation is specifically required for apoptotic cell death in mammalian cells.[15] Our finding that high levels of CPP-32 expression were associated with positive in situ end-labeling of fragmented DNA provides strong evidence of apoptotic cell death in arrhythmogenic right ventricular dysplasia.

We found that numerous cells in the right ventricle of patients with arrhythmogenic right ventricular dysplasia underwent apoptosis. The majority of these cells were morphologically identified as myocardial cells under high-power magnification. The affected areas had few or no apoptotic cells, whereas apoptotic nuclei were frequently seen in areas with little involvement. This finding suggests that the loss of myocardial cells through apoptosis is, at least in part, a primary process that precedes the filling of acellular space by fat and fibrous tissue in the absence of an inflammatory reaction.

The extent and the percentage of apoptotic myocardial cells varied among the patients. The absence of apoptosis (only one apoptotic cell per section) in two of our patients is intriguing. These patients' clinical and histologic features were similar to those of the other patients. Apoptosis may therefore not have been involved in the pathogenesis of arrhythmogenic right ventricular dysplasia in these patients. However, the possibility that most of the apoptotic cells had already been cleared by the time the heart sections were obtained cannot be ruled out. Studies of the clearance kinetics of apoptotic myocardial cells should clarify this issue.

The triggering factors for apoptotic myocardial cell death in arrhythmogenic right ventricular dysplasia remain to be elucidated. Some evidence from in vitro and in vivo studies in animals suggests that hypoxia as well as reperfusion injury are possible triggers for apoptosis in cardiomyocytes.[17,18] These factors may also contribute to the induction of apoptosis in myocardial cells of the failing canine heart.[19] Repeated ventricular arrhythmias may have produced an ischemia–reperfusion injury and contributed to the apoptotic process in our patients. The presence of myocarditis (and its related production of inflammatory cytokines) could also have had a role in the induction or aggravation of the apoptotic process. However, myocarditis is not a consistent or prominent feature of arrhythmogenic right ventricular dysplasia, and only one of our patients had associated myocarditis. Abnormal levels of resting tension, which could result from the architectural rearrangement of the myocyte compartment in the diseased right ventricle, could also have contributed to the activation of the suicide program in these cells.[20] Finally, primary abnormal control of genes involved in the regulation of programmed cell death — for instance, *CPP-32* — remains plausible.

In conclusion, we found that numerous myocardial cells from the right ventricles of a majority of patients with lethal arrhythmogenic right ventricular dysplasia actively undergo programmed cell death. This finding could account, at least in part, for the progressive loss of myocardial cells observed in this disease and may shed new light on its pathogenesis.

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